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(57) Abstract

It has now turned out that it is possible carry out a simple test for measuring a patient's ability to metabolise a certain drug by applying a method comprising the steps of a) isolating and/or providing detectable amounts of single-stranded DNA from said sample by using known methods; b) hybridising the single-stranded DNA obtained in step a) with a detection primer comprising a plurality of nucleotide residues, said primer being complementary to a target nucleotide sequence immediately adjacent and 5' in relation to a defined point mutation of a single-stranded DNA encoding a cytochrome P450 isoform, where said point mutation is known to affect said isoform's ability to metabolise said drug, such that there are no nucleotide residues between the defined point mutation and the 3' end of the detection primer that are identical to the first or second nucleotide residues of the point mutation to be detected, when the detection primer is hybridised to the target nucleic acid; c) extending the primer using a polymerising agent in a mixture comprising one or more nucleoside triphosphates wherein the mixture includes at least one nucleoside triphosphate complementary to either the first or second nucleic residue comprising means for detecting the incorporation of the nucleoside triphosphate in a nucleic acid polymer, and optionally one or more chain terminating nucleoside triphosphates; d) detecting the incorporation of the nucleoside triphosphate using said means, whereby it is determined whether said sample contains said point mutation of said cytochrome P450 isoform.

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A METHOD FOR MEASURING A PATIENT'S ABILITY TO METABOLISE CERTAIN DRUGS

The present application relates to an assay method for monitoring metabolism of certain drugs in an individual. More particularly, the invention relates to a method for determining the presence of point mutations in isoforms of cytochrome P450, which point mutations are known to affect the isoforms' abilities to metabolise said drugs. The invention also relates to primers and diagnostic kits that are suitable for carrying out the invention.

Technical background

All reference cited in the following description are incorporated into the disclosure by reference.

Single nucleotide variations have been estimated to occur in a frequency of approximately one out of thousand nucleotides in the human genome (Cooper et al., J. Hum. Genet. (1985) 69:201). Many of these mutations may not give rise to a phenotype but a great number of the genetic diseases known to date are caused by single nucleotide polymorphisms. As a consequence, detection of single nucleotide mutations in specific genes will become of increasing interest in order to understand the ethiology of many genetic diseases.

Drug metabolism involves enzymes that either oxidise (phase I) or conjugate (phase II) xenobiotics. The major route of phase I drug metabolism is maintained by a group of enzymes termed cytochrome P450 which are located in the endoplasmatic reticulum primarily in the liver (Linder et al., Clinical Chemistry (1997) 43:254). Cytochromes P450 (CYP) are comprised by a super gene family of mixed function oxidases that metabolises a large number of xenobiotics including drugs. Thirty or more of these enzymes have been characterised in the human so far, each with distinct catalytic specificity and unique regulation. Because of the diversity of these enzymes, they have been subdivided into subpopulations or isoforms based on their sequence homology, The

polymorphism of the catalytic abilities of these enzymes result in the appearance of different phenotypes with differential capacities to metabolise drugs. Extensive metabolism (EM) of a drug is characteristic of the normal population and represents the wild-type allele, poor metabolism (PM) is due to poor or no catalytic capacity by a specific enzyme, in most cases due to mutations or deletions of the gene, whereas ultra-extensive metabolism (UEM) in general is caused by gene duplications.

The most important isoforms involved in drug metabolism are CYP2D6, CYP2C9, CYP2C19 and CYP3A4. Several of these CYP isoforms are known to be polymorphic which results in differential capacities in metabolising drugs such as omeprazole (proton pump inhibitors), phenytoin (anti-convulsant), verapamil (calcium-antagonists), propanolol (beta-blockers) and many others. The CYP2C9 isoform is involved in hydroxylation of tolbutamide, phenytoin and S-warfarin among others. Specifically, CYP2C9 converts S-warfarin into the inactive phenolic metabolite S-7-hydroxywarfarin and thereby controls the pharmacological activity of this drug. Here too, polymorphisms among these enzymes exist resulting in differential capacities to metabolise drugs. The genetic basis of this polymorphism is single nucleotide mutations resulting in the expression of two allelic variants, CYP2C9*2 and CYP2C9*3. The CYP2C9*2 allele has cysteine substituted for arginine at amino acid 144 in the protein and CYP2C9*3 has leucine substituted for isoleucine at position 359. The frequencies of these alleles have been reported to be between 7 and 19 % in Caucasian populations. Although homozygous individuals for these alleles are relatively uncommon, *in vitro* studies of the metabolism of warfarin have shown impaired catalytic ability by these variant proteins (Steward et al., Pharmacogenetics (1997) 7:361). For instance, CYP2C9*3 possesses only 5% of the catalytic capacity for S-warfarin as compared to the CYP2C9 wild type enzyme.

Warfarin is a widely used anticoagulant of coumarin type which acts by blocking synthesis of the vitamin K-dependant coagulation factors II, VII, IX and X in the liver. The indications for taking S-warfarin are all diseases where prevention of extensive blood clotting is a crucial factor in the efficient treatment of patients. Examples of such

diseases are acute embolic diseases of heart, lung or brain. In these cases the treatment is often combined with heparine. More specific indications are diseases where a lifelong treatment with anti-coagulants is required. Such diseases include recurrent venous thrombosis, pulmonary embolism and chronic atrial fibrillations. The major difficulties with the use of this drug are a broad range of interactions with other drugs as well as nutritional factors. The complicated treatment of patients with this drug carries the risk of serious hemorrhage in as much as 9 % per patient year (Fihn et al., Ann. Intern. Med. (1996) **124**:970; Steward et al., Pharmacogenetics (1997) 7:361). Therefore, pretreatment evaluation of the CYP2C9 status of potential patients to be treated with warfarin would significantly reduce the risk of adverse drug reactions. Moreover, CYP2C9 metabolises the transformation of the anti-convulsant Valproic acid (VDA) into the unsaturated metabolite 4-ENE-VPA. 4_ENE-VPA acts hepatotoxic and causes several deaths yearly in the US (Sadeque et al., J. Pharmacol. Exp. Ther. (1997) **283**:698).

The CYP2C19 isoform is involved in 4-hydroxylation (or 5-hydroxylation) of tricyclic antidepressants such as imipramin, anti-malarial prodrugs as for instance proguanil and proton pump inhibitors such as omeprazole or pentaprazole (Linder et al., Clinical Chemistry (1997) **43**:254). This subfamily is polymorphogenic due to single nucleotide mutations (SNP) of the wild type allele. The M1 allele contains a G_{686}-A_{686} substitution which creates a novel aberrantly spliced CYP2C19 mRNA. This results in the production of an inactivated truncated protein. The M2 allele contains a G_{641}-A_{641} substitution resulting in a premature stop codon. Therefore, these two alleles represent poor metabolise phenotypes.

Detection of single-point mutations (SNP), such as the above mentioned mutations, can be performed using different techniques. In general, such assays can be subdivided into techniques where detection of SNP:s involves electrophoretic separation of DNA sequences and techniques using solid supports. Techniques using solid supports have several advantages as compared to electrophoretic separation techniques. Firstly, the solid-phase assays involve relatively few and simple manipulations that are amenable to full automation. Secondly, non-radioactive methods can conveniently be used in the solid

phase assays and thirdly, these assays give numerical results allowing e.g. statistical treatment.

As in the case of solid phase assays, different assay types may be distinguished. These techniques include hybridisation with sequence-specific oligonucleotide probes such as "reverse dot blot" or sandwhich hybridisation. These techniques require very careful design of the sequence-specific probes and close monitoring of reaction conditions and may thus only be performed in highly specialised laboratories. Similar problems are encountered with sequence-specific amplification which require careful optimisation of the PCR conditions. Here too, only highly specialised laboratories are capable of performing this technique. Finally, sequencing of defined DNA sequences requires a costly infrastructure and trained personnel which currently only can be provided at highly specialised laboratories.

Since the CYP2C19 and CYP2C9 enzymes metabolise a variety of drugs where relative overdosing poses a potential threat to the patient's health, there is need for a simple analytical test clarifying the genetic status of the individual prior to drug intake, as knowledge of a person's genetic status prior to drug intake could substantially reduce the risk for adverse drug reactions.

Summary of the invention

It has now turned out that it is possible carry out a simple test for measuring a patient's ability to metabolise a certain drug by applying a method comprising the steps of

a) isolating and/or providing detectable amounts of single-stranded DNA from said sample by using known methods;

b) hybridising the single-stranded DNA obtained in step a) with a detection primer comprising a plurality of nucleotide residues, said primer being complementary to a target nucleotide sequence immediately adjacent and 5' in relation to a defined point mutation of a single-stranded DNA encoding a cytochrome P450 isoform, where said point mutation is known to affect said isoform's ability to metabolise said drug, such

that there are no nucleotide residues between the defined point mutation and the 3' end of the detection primer that are identical to the first or second nucleotide residues of the point mutation to be detected, when the detection primer is hybridised to the target nucleic acid;

c) extending the primer using a polymerising agent in a mixture comprising one or more nucleoside triphosphates wherein the mixture includes at least one nucleoside triphosphate complementary to either the first or second nucleic residue comprising means for detecting the incorporation of the nucleoside triphosphate in a nucleic acid polymer, and optionally one or more chain terminating nucleoside triphosphates;

d) detecting the incorporation of the nucleoside triphosphate using said means, whereby it is determined whether said sample contains said point mutation of said cytochrome P450 isoform.

Detailed description of the invention

The solid phase mini-sequencing technique disclosed in WO91/13075 provides a cheap and robust assay which can be performed by any laboratory equipped with a thermal cycler. Moreover, this technique does not require any specially trained personnel. Furthermore, the solid phase mini-sequencing technique does not require radioactively labeled nucleotides. Therefor it exhibits higher safety standards than such techniques. Finally, this technique provides an excellent possibility of detecting either homozygote or heterozygote alleles within a defined sample.

Accordingly, the present invention relates to a method for determining the ability of cells in a sample to metabolise a certain drug comprising detecting a defined point mutation of a single-stranded DNA encoding a cytochrome P450 isoform, where said point mutation is known to affect said isoform's ability to metabolise said drug.

In another embodiment the present invention relates to detection primers useful in the above mentioned method, which primers hybridise to target nucleotide sequences

immediately adjacent and 5' in relation to a point mutation of a DNA, said DNA encoding an isoform of cytochrome P450.

In yet another embodiment, the present invention relates to a diagnostic kit for carrying out said method, said kit comprising at least one detection primer as defined above, at least two amplification primers derived from a sequence encoding a cytochrome P450 isoform , said amplification primers being chosen in such a way that a subsequence of said cytochrome P450-encoding sequence to which said detection primer hybridises is amplified, and a DNA-polymerising agent.

As disclosed herein, the term "drug" relates to drugs that are metabolised by cytochrome P450 isoforms. Examples of such drugs are omeprazole , pentaprazole, phenytoin, verapamil, propanolol, tolbutamide, S-warfarin, tricyclic antidepressants such as imipramin and anti-malarial prodrugs such as proguanil.

As disclosed herein, the term "detection primer" relates to an oligonucleotide which hybridises to a site immediately adjacent 5' in relation to a defined point mutation. The term "amplification primer" relates to one of two primers forming a primer pair that is used according to well-known amplification procedures such as PCR. Both detection primers and amplification primers according to the invention comprises 8 - 70 nucleotides, preferably 10-30 nucleotides, and most preferably 15 - 25 nucleotides.

As disclosed herein, the term "affinity pair" relates to a pair of chemical, preferably biochemical, compounds that binds specifically and strongly to each other. Examples of such pairs include, but are not limited to antibody-antigen, biotin-avidin/streptavidin, enzyme-substrate, a pair of complementary oligonucleotides, protein A-IgG, etc.

As disclosed here, the term "polymerising agent" relates to a DNA polymerising agent. An example of such an gent is the Klenow fragment of Escherichia coli DNA polymerase I, but any DNA polymerase can be used in the method of this invention.

According to the present invention, the presence of point mutations can be detected by adding labelled nucleotides to the detection primer. Any kind of detectable labels, such as one member of an affinity pair, radioactive nuclides, fluorescent compounds, enzymes inducing light emissions or colour changes etc. can be bound to an ordinary nuclotide in order to obtain a labelled nucleotide. Alternatively, it is possible to use modified nucleotides such as chain-terminating dideoxynucleotides. The skilled person is well aware of how to choose suitable labelled nucleotides as well as how to choose suitable detection procedures when carrying out the method according to the present invention.

The present invention will now be further described with reference to the enclosed figure ande tables, in which:

fig. 1 discloses a photo of an electrophoresis gel where lanes A-E represent the following PCR products: A: CYP2C9*2 (simplex PCR), B: CYP2C9*3 (simplex PCR), C: CYP2C9*2*3 (20 µl*3, multiplex PCR), D: CYP2C9*2*3 (15 µl * 3, multiplex PCR), E: CYP2C9*2*3 (10 µ * 3, multiplex PCR). In the multiplex PCR

constant primer concentrations for the CYP2C9*2 allele and decreasing concentrations of primers for the CYP2C9*3 allele were used in order to optimise the multiplex PCR conditions;

table 1 discloses results obtained when the PCR products shown in fig. 1 have been subjected to minisequencing reactions. Both specific and non-specific sequence primers as well as complimentary or non-complimentary nucleotides have been used. The figures shown in table 1 represent optical density (OD) values from an ELISA determined at 405 nm. The table shows which PCR products were coated onto the streptavidin-coated ELISA plate (columns), which sequence primers were used (rows) and which nucleotides were used in the sequencing reaction (columns);

table 2 shows the calculated ratio of the OD at 405 nm from nucleotides incorporated by the mini-sequencing reaction. The ratio values presented in this table have been

calculated from the OD values in table 1. The ratio was calculated as follows: *complementary nucleotide (OD at 405 nm/ complementary nucleotide + non-complementary nucleotide (OD at 405 nm).* A ratio of > 0.85 is significant for an incorporation of complementary nucleotides when using homozygous alleles.

Experimental procedures

The mini-sequencing technique is based on amplification of defined genes with PCR (Polymerase Chain Reaction) using biotinylated or otherwise conjugated oligonucleotides (primers). In general, where possible a multiplex amplification procedure is utilised. Following amplification, the biotinylated PCR products are immobilised on streptavidin-coated microwell plates and the PCR products are sequenced using an allele-specific oligonucleotide. Possible mutations within the immobilised PCR product representing a defined allele are detected by incorporation of a mutation-specific labelled nucleotide. Incorporatio of a complementary nucleotide can be detected either directly or indirectly utilising various established detection methods. Using this technique, it is possible to detect homozygote or heterozygote alleles based on single nucleotide mutations within an individual.

Genomic DNA can be prepared using any established method described in the literature (PCR Protocols, Innis MA et al., Academic Press 1990; PCR, a practical approach, McPherson, MJ et al., Oxford University Press, 1991) or using any DNA purification kit present. The DNA prepared in these experiments has been prepared using the QIAamp Blood Kit (Qiagen Inc, USA) according to the description provided by the manufacturer. Genomic DNA can be prepared using any sample-containing nucleated cells. The typical yield using the above mentioned DNA purification kit is 10 ng/µl. 250 ng of purified genomic DNA was used as a template in the subsequent PCR. The primers used for the PCR reactions are described in the sequence listing as SEQ.ID.Nos 4, 5, 7, 8, 10, 11, 13 and 14. In the following description of the experimental procedures for the PCR and mini-sequencing method of CYP genes, primers specificfor the CYP2C9 alleles were

used. The same experimental procedures as described below were used when CYP2C19 alleles were studied with the exception for using CYP2C19-specific oligonuclotides.

A 2x mastemix for the multiplex PCR of CYP2C9 alleles was prepared as follows:

- Tris-HCl (100 mM, pH8.8)
- $(NH_4)_2SO_4$ (30 mM)
- Triton X-100 (0.2 % vol./vol.)
- Gelatin (0.02% wt/vol.)
- dNTPs (0.4 μM)
- SEQ.ID.NO. 4, 5, 7, 8 (0.4 mM of each)
- $MgCl_2$ (3.0 mM)
- ddH_2O up to 500 μl

For the PCR reaction 50 μl of the above described 2x mastermix was subject to a PCR tube (thin wall PCR tubes, Perkin-Elmer Inc. USA). 24,5 μl of ddH2O, O,5 μl Taq-polymerase (2,5 Units, Perkin-Elmer, Inc, USA) and 25 μl geonomic DNA (250 ng) was added to the tube and the reaction mix was overlaid with 50 μl mineral oil.

The thermal conditions for amplification of the CYP2C9 alleles were as follows: An initial denaturation step at 96°C for 2 minutes thereafter 96°C (30 sec), 60°C (30 sec) (58°C for the CYP2C19 alleles) and 72°C (30 sec), 35 cycles. Following the PCR amplification, 100 μl of the amplified sample was mixed with 400 μl of Binding buffer (buffer 1) containing 20 mM sodium phosphate, pH 7.5, 100 mM NaC1 and 0.1 % (v/v) Tween-20.

50 μl aliquots were subsequently transferred to streptavidin coated said phase, such as microwell plates (MWP) which are commercially available (Labsystems, Helsinki, Finland). The MWP were then incubated at 22°C for 15 minutes. Following incubation, the immobilized PCR samples were denatured using a denaturing solution containing NaOH (50 mM) for 1 minute at 22°C. The MWP were washed using a buffer (buffer 2)

containing Tris-HC1 (40 mM, pH 8.8), EDTA (1mM), NaC1 (50 mM) and Tween-20 (0.1%).

For the minisequencing reaction, every well of the MWP was incubated with an appropriate minisequencing primer (final concentration 0.1 µM) diluted in 5 µl of 10 x DNA polymerase buffer (buffer 3) containing Tris-HCl (500 mM, pH 8.8), $(NH_4)_2SO_4$ (150 mM), $MgC1_2$ (15mM), Triton X-100 (1% V/V), Gelatin (0.1%W/V), DNA polymerase (0.1 units), flourescein-12-dNTP complementary to the nucleotide to be detected (final concentration of 0.1 µM) and ddH_2O to a final volume of 50 µl.

The MWP were incubated at 55° C for 30 minutes. Following the minisequencing reaction the MWP were washed using buffer 2. Incorporated nucleotides were detected using alkaline phosphatase (AP) conjugated anti-FITC monoclonal antibodies (0.75 U/ml) diluted in a buffer (buffer 4) containing : Hepes (25 mM), NaC1 (125 mM), $MgCl_2$ (2 mM), BSA (1%) and Tween-20 (0.3 % V/V). Incubation was done at 22° C for 15 minutes and the plates were subsequently washed using buffer 2. Detection of bound monoclonal antibodies was performed by incubation the MWP using a detection buffer (buffer 5) containing diethanolamine (10.6 % W/V), $MgC1_2$ (0.05 % W/V) and para-nitro-phenyl phosphate (4 mg/ml) for 20 minutes at 22° C. Detection of incorporated dNTP's was done at 405 nm using a commercially available spectrophotometer.

RESULTS

PCR amplification and minisequencing has been performed using CYP2C9 and CYP2C19 specific primers. The results shown below demonstrate amplification and minisequencing of the CYP2C9 alleles. Human genomic DNA was purified as described in the method section. 250 ng of genomic DNA was subjected to PCR as described above. The results of a representative experiment are demonstrated in figure 1.

Next a minsequencing reaction of the amplified DNA was done as decribed above. By using alleles specific sequencing primers incorporated dNTP's could be detected in subsequent detection steps as described above. These results are shown in table 1.

In order to obtain mumerical values a ratio of the OD was calculated based on the formula shown below. These results are demonstrated in table 2.

These results clearly show that by using gene-specific primers it was possible to amplify and sequence cytochrome P450 specific alleles using PCR and the minisequencing technique as described above.

TABLE 1

Sequence primers	*CYP2C9*2 samples*		*CYP2C9*3 samples*		*2*3 20 µl *3 multiples*		*2*3 15 µl *3 multiples*		*2*3 10 µl *3 multiples*		*No coating*	
CYP2C9*2 sek	2.211	C	0.186	G	0.697	C	0.702	C	0.615	G	0.137	G
CYP2C9*2 sek	0.157	U	0.161	U	0.191	U	0.201	U	0.312	U	0.138	U
CYP2C9*3 sek	0.151	C	0.191	G	0.198	G	0.192	G	0.239	G	0.139	G
CYP2C9*3 sek	0.164	U	1.220	U	0.694	U	0.818	U	0.697	U	0.137	U
CYP2C9*2 sek	0.186	G	0.141	C	0.179	A	0.222	A	0.161	A	0.139	A
CYP2C9*2 sek	0.153	A	0.147	A	0.183	A	0.224	A	0.167	A	0.140	A
CYP2C9*3 sek	0.164	G	0.153	C	0.265	A	0.302	A	0.200	A	0.137	A
CYP2C9*3 sek	0.147	A	0.336	A	0.255	A	0.285	A	0.223	A	0.129	A

TABLE 2

PCR Primers	*Nucleotide*	*Ratio*
CYP2C9*2	C	0.99
CYP2C9*3	U	0.97
CYP2C9*2*3 (20 µl)	C	0.95
CYP2C9*2*3 (20 µl)	U	0.95
CYP2C9*2*3 (15 µl)	C	0.92
CYP2C9*2*3 (15 µl)	U	0.94
CYP2C9*2*3 (10 µl)	C	0.76
CYP2C9*2*3 (10 µl)	U	0.72

Claims

1. A method for determining the ability of cells in a sample, to metabolise a certain drug, comprising the steps of

a) isolating and/or providing detectable amounts of single-stranded DNA from said sample by using known methods;

b) hybridising the single-stranded DNA obtained in step a) with a detection primer comprising a plurality of nucleotide residues, said primer being complementary to a target nucleotide sequence immediately adjacent and 5' in relation to a defined point mutation of a single-stranded DNA encoding a cytochrome P450 isoform, where said point mutation is known to affect said isoform's ability to metabolise said drug, such that there are no nucleotide residues between the defined point mutation and the 3' end of the detection primer that are identical to the first or second nucleotide residues of the point mutation to be detected, when the detection primer is hybridised to the target nucleic acid;

c) extending the primer using a polymerising agent in a mixture comprising one or more nucleoside triphosphates wherein the mixture includes at least one nucleoside triphosphate complementary to either the first or second nucleic residue comprising means for detecting the incorporation of the nucleoside triphosphate in a nucleic acid polymer, and optionally one or more chain terminating nucleoside triphosphates;

d) detecting the incorporation of the nucleoside triphosphate using said means, whereby it is determined whether said sample contains said point mutation of said cytochrome P450 isoform.

2. A method according to claim 1, **characterised** in that the single-stranded DNA isolated and/or provided in step a) is obtained by performing a modified amplification reaction in which one of the two amplification primers comprises a first attachment moiety bound to the primer, thereby obtaining a double-stranded amplification product in which only one of the strands comprises a first attachment moiety, where said first attachment moiety is one half of an affinity pair DEFINIERA, and then simultaneously or sequentially in any order rendering the amplification product single-stranded and

immobilising the strand comprising the first attachment moiety to a solid support with the aid of the other component of the affinity pair, whereafter all unbound material is removed.

3. A method according to anyone of claim 1 or claim 2, **characterised** in that said point mutation to be detected only comprises one altered nucleotide.

4. A detection primer hybridising to a target nucleotide sequence immediately adjacent and 5' in relation to a point mutation of a single stranded DNA encoding cytochrome P450 isoform CYP2C19, said primer consisting of a subsequence of 10 - 70 nucleotides of the sequence:

5' GTTCTTTTAC TTTCTCCAAA ATATCACTTT CCATAAAAGC AAGGTTTTTA

AGTAATTTGT TATGGCTTCC 3'

which subsequence always comprises the nucleotide located in the 3' end of the sequence above.

5. A detection primer according to claim 4 consisting of a subsequence of 10-30 nucleotides.

6. A detection primer according to claim 5 which is 5'-AAGTAATTTGTTATGGGTTCC-3'.

7. A detection primer hybridising to a target nucleotide sequence immediately adjacent and 5' in relation to a point mutation of a single stranded DNA encoding cytochrome P450 isoform CYP2C19, said primer consisting of a subsequence of 10 - 70 nucleotides of the sequence:

5'-TTGAATGAAA ACATCAGGAT TGTAAGCACC CCCTGAATCC AGATATGCAA

TAATTTTCCC ACTATCATTG ATTATTTCCC-3'

which subsequence always comprises the nucleotide located in the 3' end of the sequence above.

8. A detection primer hybridising to a target nucleotide sequence immediately adjacent and 5' in relation to a point mutation of a single-stranded DNA encoding cytochrome P450 isoform CYP2C19, said primer consisting of a subsequence of 8 - 50 nucleotides of the sequence:

5'-AACTTGATGG AAAAATTGAA TGAAAACATC AGGATTGTAA GCACCCCCTG-3'

which subsequence always comprises the nucleotide located in the 3' end of the sequence above.

9. A detection primer according to claim 8 which is: 5'-GATTGTAAGCACCCCCTG-3'.

10. A detection primer hybridising to a target nucleotide sequence immediately adjacent and 5' in relation to a point mutation of a single-stranded DNA encoding cytochrome P450 isoform CYP2C9, said primer consisting of a subsequence of 8 -50 nucleotides of the sequence:

5'-CCCTCATGAC GCTGCGGAAT TTTGGGATGG GGAAGAGGAG CATTGAGGAC-3'

which subsequence always comprises the nucleotide located in the 3' end of the sequence above.

11. A detection primer according to claim 10 which is: 5'-AAGAGGAGCATTGAGGAC-3'.

12. A detection primer hybridising to a target nucleotide sequence immediately adjacent and 5' in relation to a point mutation of a single-stranded DNA encoding cytochrome P450 isoform CYP2C9, said primer consisting of a subsequence of 8 - 50 nucleotides of the sequence:

5'-CTTGGTTTTT CTCAACTCCT CCACAAGGCA GCGGGCTTCC TCTTGAACAC-3'

which subsequence always comprises the nucleotide located in the 3'end of the sequence above.

13. A detection primer hybridising to a target nucleotide sequence immediately adjacent and 5' in relation to a point mutation of a single-stranded DNA encoding cytochrome P450 isoform CYP2C9, said primer consisting of a subsequence of 8-50 nucleotides of the sequence:

5'-TTTAATGTCA CAGGTCACTG CATGGGGCAG GCTGGTGGGG AGAAGGTCAA-3'

which subsequence always comprises the nucleotide located in the 3' end of the sequence above.

14. A detection primer according to claim 13, which is: 5'-TGGTGGGGAGAAGGTCAA-3'.

15. A detection primer hybridising to a target nucleotide sequence immediately adjacent and 5' in relation to a point mutation of a single-stranded DNA encoding cytochrome P450 isoform CYP2C9, said primer consisting of a subsequence of 8-50 nucleotides of the sequence:

5'-GGAGCCACAT GCCCTACACA GATGCTGTGG TGCACGAGGT CCAGAGATAC-3'

which subsequence always comprises the nucleotide located in the 3' end of the sequence above.

16. 16 A kit for detecting a possible point mutation in cytochrome P450 isoform CYP2C19 comprising:

a) a detection primer according to anyone of claims 4-9;

b) two amplification primers derived from the sequence according to SEQ.ID.NO. 1 and a sequence complementary to SEQ.ID.NO. 1, said primers being chosen in such a way

that a subsequence of the sequence according SEQ.ID.NO.1 containing said possible point mutation and a sequence complimentary to said detection primer can be amplified;

c) at least one labelled nucleoside triphosphate; and

d) a DNA polymerasing agent.

17. . A kit for detecting a possible point mutation in cytochrome P450 isoform CYP2C9 comprising:

a) a detection primer according to anyone of claims 10-12;

b) two amplification primers derived from the sequence according to SEQ.ID.NO. 2 and a sequence complementary to SEQ.ID.NO. 2, said primers being chosen in such a way that a subsequence of the sequence according SEQ.ID.NO.2 containing said possible point mutation and a sequence complimentary to said detection primer can be amplified;

c) at least one labelled nucleoside triphosphate; and

d) a DNA polymerasing agent.

18. A kit for detecting a possible point mutation in cytochrome P450 isoform CYP2C9 comprising:

a) a detection primer according to anyone of claims 13-15;

b) two amplification primers derived from the sequence according to SEQ.ID.NO. 3 and a sequence complementary to SEQ.ID.NO. 3, said primers being chosen in such a way that a subsequence of the sequence according SEQ.ID.NO.3 containing said possible point mutation and a sequence complimentary to said detection primer can be amplified;

c) at least one labelled nucleoside triphosphate; and

d) a DNA polymerasing agent.

FIG. 1

A: CYP2C9*2, **B**: CYP2C9*3, **C**: CYP2C9*2*3 (20 µl)*3,
D: CYP2C9*2*3 (15 µl*3), **E**: CYP2C9*2*3 (10 µl*3)

<213> Homo sapiens

<400> 5
atcaataaag tcccgagggt 20

<210> 6
<211> 21
<212> DNA
<213> Homo sapiens

<400> 6
aagtaatttg ttatgggttc c 21

<210> 7
<211> 20
<212> DNA
<213> Homo sapiens

<400> 7
tgcaatgtga tctgctccat 20

<210> 8
<211> 20
<212> DNA
<213> Homo sapiens

<400> 8
gttcagggct tggtcaatat 20

<210> 9
<211> 18
<212> DNA
<213> Homo sapiens

<400> 9
gattgtaagc acccccctg 18

<210> 10
<211> 21
<212> DNA
<213> Homo sapiens

<400> 10
cctagtttcg tttctcttcc t 21

<210> 11
<211> 21
<212> DNA

<213> Homo sapiens

<400> 11
cagtgatatg gagtagggtc a 21

<210> 12
<211> 18
<212> DNA
<213> Homo sapiens

<400> 12
aagaggagca ttgaggac 18

<210> 13
<211> 20
<212> DNA
<213> Homo sapiens

<400> 13
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<210> 14
<211> 20
<212> DNA
<213> Homo sapiens

<400> 14
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<400> 15
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<210> 16
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<211> 18
<212> DNA

<213> Homo sapiens

<400> 17
cacgaggtcc agagatac 18

<210> 18
<211> 19
<212> DNA
<213> Homo sapiens

<400> 18
ctatcattga ttatttccc 19

<210> 19
<211> 22
<212> DNA
<213> Homo sapiens

<400> 19
gaaaattatt gcatatctgg at 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/01449

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12Q 1/68, C12Q 1/34, C12N 15/11 // C12N 9/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12Q, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9530766 A1 (THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES), 16 November 1995 (16.11.95), page 40, line 27 - line 35; page 42, line 37; page 45, line 14 - line 16	16
A	--	1-9
A	WO 9113075 A2 (ORION-YHTYMÄ OY), 5 Sept 1991 (05.09.91), especiale page 4, line 28 - page 5, line 30 and claims	1-18
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 December 1999	17 January 2000 (17.01.00)
Name and mailing address of the ISA Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson / MR Telephone No. +46 8 782 25 00

Form PCT ISA 210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/01449

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	File WPI, Derwent accession no. 98138246, SRL KK: "Detecting point mutation in exon 4 of human cytochrome P450 2C19 gene - comprises carrying out PCR using specific oligio:nucleotide primers, useful for, e.g. detecting abnormalities in S-mephenytoin metabolism"; & JP,a,10014585, 980120,DW9813	8-9
	--	
A	Pharmacogenetics, Volume 6, 1996, Michael J. Stubbins et al, ""Genetic analysis of the human cytochrome P450 CYP2C9 locus"" page 429 - page 439	10-15,17-18
	--	
A	WO 9534679 A2 (THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES), 21 December 1995 (21.12.95)	10-15,17-18
	--	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/01449

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99 01449

According to PCT rule 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features", i.e., features that define a contribution which each of the inventions, considered as a whole, makes over the prior art.

The application relates to a method and different DNA-sequences (detection primers) from isoforms of cytochrome P450. A special technical feature could be detection primers hybridising immediately adjacent and in 5' in relation to a point mutation in a DNA sequence encoding cytochrome P450 isoforms. However, such primers are contemplated in WO95/30766, see e.g. page 40, lines 30-35. Thus, the claimed invention fails to comply with the demand for unity *a posteriori* .

The following inventions were found:

Invention 1, claims 1-3: Method for determining the ability of cells in a sample to metabolise certain drugs comprising the steps a)-d) in claim 1.

Invention 2, claims 4-6: Detection primers derived from the sequence in claim 4.

Invention 3, claims 7: Detection primers derived from the sequence in claim 7.

Invention 4, claims 8-9: Detection primers derived from the sequence in claim 8.

Invention 5, claims 10-11 and 17: Detection primers derived from the sequence in claim 10 and a kit comprising the primers

Invention 6, claims 12 and 17: Detection primers derived from the sequence in claim 12 and a kit comprising the primers.

Invention 7, claims 13-14 and 18: Detection primers derived from the sequence in claim 13 and a kit comprising the primers.

Invention 8, claims 15 and 18: Detection primers derived from the sequence in claim 15 and a kit comprising the primers.

Invention 9, claim 16: A kit for detecting a possible point mutation in the cytochrome P450 isoform CYP2C19 comprising a detection primer according to claims 4-9.

In spite of the non-unity all inventions has been searched.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/12/99

International application No.
PCT/SE 99/01449

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9530766 A1	16/11/95	AU 2476695 A	29/11/95
		US 5912120 A	15/06/99
WO 9113075 A2	05/09/91	AT 180019 T	15/05/99
		AU 642709 B	28/10/93
		AU 7235191 A	18/09/91
		CA 2071537 A	17/08/91
		DE 648280 T	30/11/95
		DE 69131233 D,T	04/11/99
		EP 0648280 A,B	19/04/95
		SE 0648280 T3	
		ES 2072235 T	16/07/95
		FI 102297 B	00/00/00
		FI 923653 A	14/08/92
		GR 95300047 T	31/07/95
		HU 211058 B	30/10/95
		IL 97222 A	31/08/95
		JP 2786011 B	13/08/98
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		NO 923116 A	10/08/92
		NZ 237134 A	25/06/92
		PT 96776 A,B	31/10/91
WO 9534679 A2	21/12/95	AU 2860295 A	05/01/96
		GB 2303853 A,B	05/03/97
		GB 9412054 D	00/00/00
		GB 9502728 D	00/00/00
		GB 9626479 D	00/00/00
		US 5891633 A	06/04/99
		DE 69507636 D,T	05/08/99
		EP 0795158 A,B	17/09/97
		PL 320020 A	01/09/97
		GB 9507640 D	00/00/00